Virology **295,** 97–107 (2002)
doi:10.1006/viro.2002.1360, available online at http://www.idealibrary.com on IDEAL®

w metadata, citation and similar papers at core.ac.uk

brought to you by CO

provided by Elsevier - Publisher Conn

Reorganization of Nuclear Domain 10 Induced by Papillomavirus Capsid Protein L2

Luise Florin,* Frank Schäfer,* Karl Sotlar,† Rolf E. Streeck,* and Martin Sapp*,[1]

*Institute for Medical Microbiology and Hygiene, University of Mainz, 55101 Mainz, Germany; and †Department of Pathology, University Hospital Tübingen, 72072 Tübingen, Germany

Received October 31, 2001; returned to author for revision November 28, 2001; accepted January 6, 2002

Nuclear domains (ND) 10 are associated with proteins implicated in transcriptional regulation, growth suppression, and apoptosis. We now show that the minor capsid protein L2 of human papillomavirus (HPV) type 33 induces a reorganization of ND10-associated proteins. Whereas the promyelocytic leukemia protein, the major structural component of ND10, was unaffected by L2, Sp100 was released from ND10 upon L2 expression. The total cellular amount of Sp100, but not of Sp100 mRNA, decreased significantly, suggesting degradation of Sp100. Proteasome inhibitors induced the dispersal of Sp100 and inhibited the nuclear translocation of L2. In contrast to Sp100, Daxx was recruited to ND10 by L2 expression. Coimmunoprecipitation demonstrated interaction of the two proteins. L2-induced reorganization of ND10 was observed both in cell culture and in natural HPV lesions. The differential change in protein composition observed provides further evidence to suggest that the ND10-associated proteins are an important interface of viral life cycle and host cell. © 2002 Elsevier Science (USA)

Key Words: papillomavirus; L2; ND10; Sp100; Daxx.

INTRODUCTION

The structural and functional organization of the cell nucleus of higher eukaryotes is still not well understood. In addition to accommodating the genomic chromatin, the nucleus contains a number of nonchromatin structures, including nucleoli and nuclear bodies (Lamond and Earnshaw, 1998). One class of nuclear bodies became initially known as a target of autoantibodies in patients with primary biliary cirrhosis (Sternsdorf *et al.,* 1997; Szostecki *et al.,* 1990). This permitted the identification of Sp100, a major constituent of these subnuclear structures. Sp100 and its variants interact with members of nonhistone chromatinal proteins and have been shown to influence transcription and chromatin dynamics (Seeler *et al.,* 1998, 2001). Another major component was later found to be the promyelocytic leukemia (PML) protein, which was originally discovered as the fusion partner of the retinoic acid receptor α-oncogenic fusion protein in patients with acute promyelocytic leukemia (de The *et al.,* 1990; Dyck *et al.,* 1994; Goddard *et al.,* 1991; Kakizuka *et al.,* 1991; Weis *et al.,* 1994). Subsequently these nuclear bodies were termed PML oncogenic domains or nuclear dots (ND) 10 (Ascoli and Maul, 1991). PML is the major structural component of ND10 (Ishov *et al.,* 1999; Zhong *et al.,* 2000a) and seems to be strongly associated with the nuclear matrix (Sternsdorf *et al.,* 1997). Several other proteins in addition to Sp100 and PML have been found to be associated with ND10 (Maul *et al.,* 2000; Seeler and Dejean, 1999; Negorev and Maul, 2001). These include the ubiquitin-related protein SUMO-1, which can modify PML and Sp100 through covalent modification, the Fas-modulating protein Daxx, which has also been shown to affect gene expression, transcription factors (Sp1, CBP), tumor suppressors (p53, pRb), proteasome components, proto-oncogenes, and still others (Quignon *et al.,* 1998; Sternsdorf *et al.,* 1997; Wang *et al.,* 1998; Zhong *et al.,* 2000a).

The function of ND10 has remained largely enigmatic. Given the multitude of proteins that may associate with these structures, albeit some only transiently, ND10 are unlikely to serve any single specific function. They have been proposed to be deposits of nuclear factors and have been implicated in various cellular functions including transcriptional regulation (Doucas, 2000; Li and Chen, 2000; Li *et al.,* 2000a), growth suppression (Gottifredi and Prives, 2001), and apoptosis (Quignon *et al.,* 1998; Torii *et al.,* 1999; Wang *et al.,* 1998; Zhong *et al.,* 2000b). ND10 are dynamic structures that can vary in number and size depending on cell type. They appear to be associated with the nuclear matrix and are cell cycle-regulated. The ND10 organization may change in neoplastic and inflammatory diseases or other cellular stresses, and ND10-associated proteins are upregulated by interferons (Guldner *et al.,* 1992; Maul *et al.,* 2000; Sternsdorf *et al.,* 1997; Everett, 2001). The more recent interest in ND10, however, is due to their alteration in pathological situations like virus infection.

[1] To whom correspondence and reprint requests should be addressed at Institute for Medical Microbiology and Hygiene, Johannes-Gutenberg-Universität Mainz, Hochhaus am Augustusplatz, D-55101 Mainz, Germany. Fax: +49-6131-393-2359. E-mail: sapp@mail.uni-mainz.de.

0042-6822/02 $35.00
© 2002 Elsevier Science (USA)
All rights reserved.

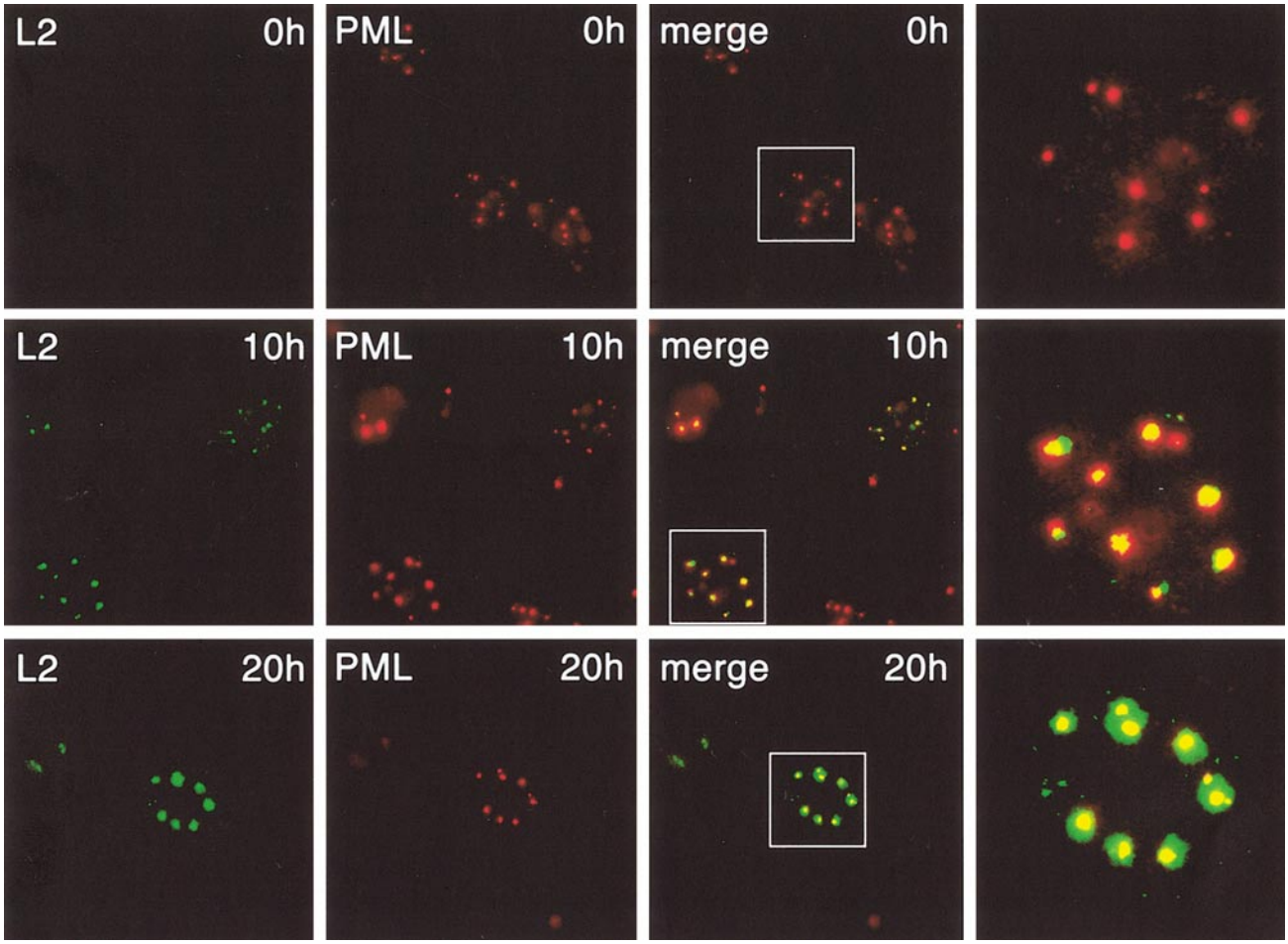

FIG. 1. L2 does not alter nuclear localization of PML. HuTK$^-$ cells were grown on coverslips and subsequently coinfected for the indicated times with VTF7-3 and vac33L2. Cells were stained for L2 and PML and visualized by DTAF- and Cy3-coupled secondary antibodies, respectively. The indicated sections have been enlarged to more clearly demonstrate colocalization.

Specific proteins of a number of DNA viruses, including herpes simplex virus (HSV), cytomegalovirus (CMV), Epstein–Barr virus (EBV), and adenovirus, are targeted to ND10 (Adamson and Kenney, 2001; Ishov and Maul, 1996; Sternsdorf *et al.,* 1997). The HSV ICPO, the CMV IE1, and the EBV BZLF1 proteins specifically abrogate the SUMO-1 modification of PML and/or Sp100, thereby inducing a complete disruption of the ND10 structure (Ahn *et al.,* 1998; Everett *et al.,* 1998; Everett and Maul, 1994; Korioth *et al.,* 1996; Muller and Dejean, 1999; Parkinson and Everett, 2000). The E4orf3 protein of adenovirus associates with PML and causes a dramatic reorganization of ND10 (Carvalho *et al.,* 1995). T-antigen of simian virus 40 and nuclear antigen-5 of EBV have also been found in association with ND10, as well as the E1 and E2 proteins of human papillomavirus (HPV) (Swindle *et al.,* 1999; Szekely *et al.,* 1996; Tang *et al.,* 2000). All of these proteins are early proteins, which suggests that ND10 may play a role in the initiation of DNA replication of these viruses. Recently the minor capsid protein (L2) of bovine papillomavirus type 1 (BPV1) was found to accumulate in ND10 and to recruit the major capsid protein (L1) into these structures (Day *et al.,* 1998). This suggests that ND10 may also be a site of encapsidation of the viral genome.

Papillomaviruses are a large group of DNA viruses of higher eukaryotes that specifically infect epithelial cells and replicate in terminally differentiating keratinocytes. The 8-kb genome associates with histones to form a minichromosome that is packaged into the viral capsid (for review see Howley (1996)). The capsid is organized into 72 capsomeres, pentamers of the major capsid protein L1, and contains several copies, possibly 12, of the minor capsid protein L2 (Baker *et al.,* 1991; Trus *et al.,* 1997). The function of L2 is not known exactly, but it has been shown to be required both for infection and for packaging of the viral DNA (Roden *et al.,* 1996; Unckell *et al.,* 1997).

In this study we have analyzed the interaction of ND10 with the minor capsid protein of HPV33, a human papillomavirus associated with malignant carcinoma (Beaudenon *et al.,* 1986). We show that this interaction induces

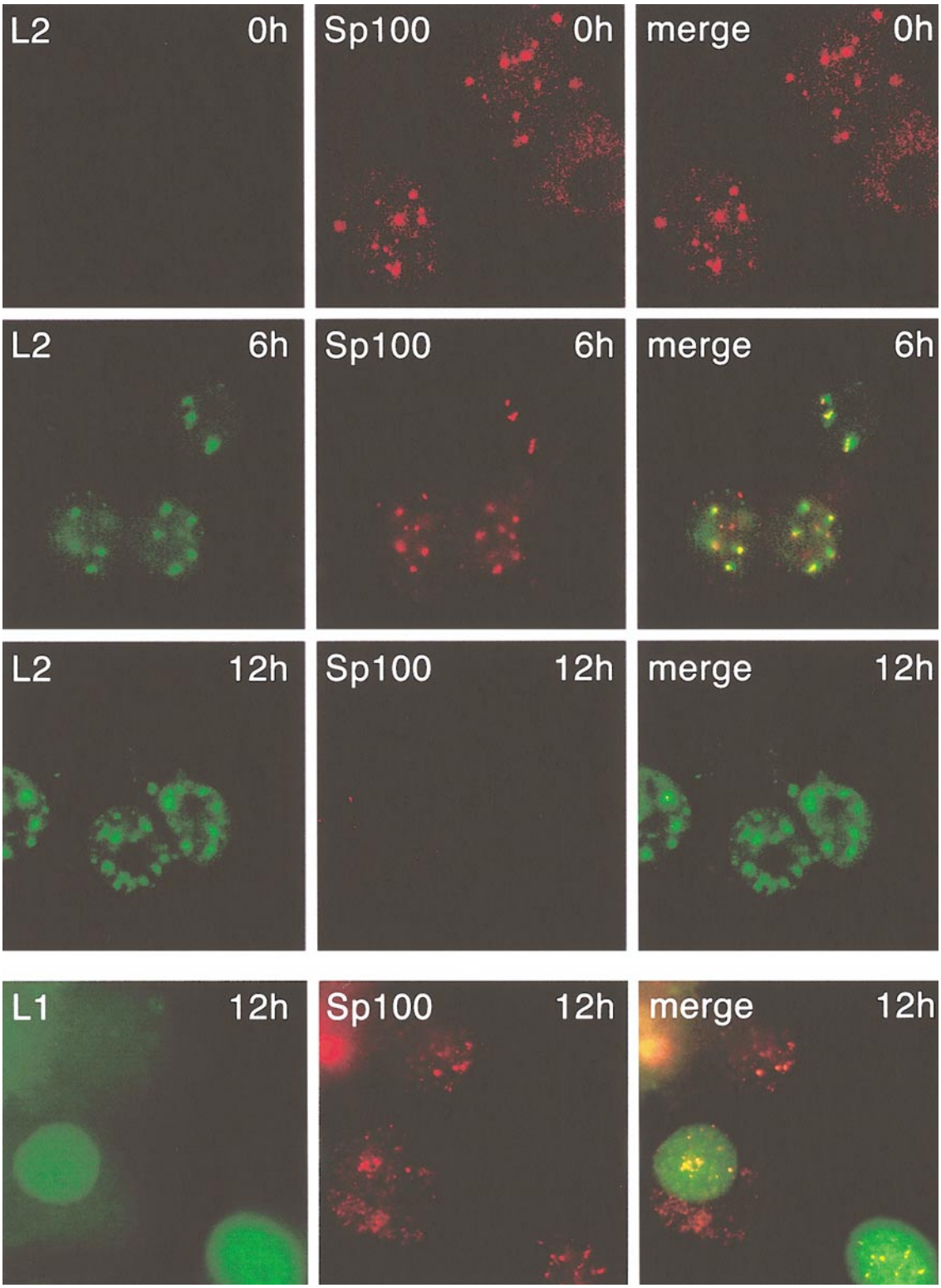

FIG. 2. L2-induced release of Sp100 from ND10. HuTK$^-$ cells were grown on coverslips and subsequently coinfected with VTF7-3 and vac33L2 or with VTF7-3 and vac33L1 for the indicated periods of time. Cells were stained for L2 and Sp100 or for L1 and Sp100 and visualized by DTAF- and Cy3-coupled secondary antibodies, respectively.

major changes of ND10 affecting proteins that are tightly associated with the nuclear matrix and that are involved in transcriptional regulation. This provides for the first time insight into the close link between this late viral protein, nuclear subdomains, and regulators of transcriptional repression and chromatin structure.

RESULTS

Association of ND10 with L2 does not affect PML

To study the association of ND10 with the papillomavirus minor capsid protein, L2, we visualized L2 and ND10-associated proteins in human cell line HuTK$^-$ by indirect immunofluorescence. Similar results were obtained with HeLa and KH cells. L2 was expressed using vaccinia virus vac33L2 and helper virus VTF7-3 encoding HPV33 L2 and T7 RNA polymerase, respectively. L2 expression was first detected 4 h after infection by diffuse nuclear staining (not shown). Ten hours after infection it localized mainly to ND10, which were identified by immunostaining of PML (Fig. 1). Another 10 h later the L2-positive speckles had significantly expanded, suggesting further accumulation. No change in the number and intensity of PML foci could be detected in the course of L2 expression (Fig. 1). This indicates that the core of ND10, supposed to be formed by PML oligomers (Ishov *et al.,* 1999; Zhong *et al.,* 2000a), is unaffected by L2. L2 colocalized with PML and continued to be deposited around the PML cores as shown in the higher magnification (Fig. 1).

Sp100 is released from ND10 by L2 expression

Another major constituent of ND10 besides PML is the Sp100 protein. It has been reported to be firmly bound to the nuclear matrix but not to PML and to exist in various isoforms (Koken *et al.,* 1994; Sternsdorf *et al.,* 1997; Seeler *et al.,* 2001). Prior to infection of HuTK$^-$ cells with vaccinia virus, Sp100 was detected in ND10 (Fig. 2) where it colocalized with PML, in agreement with previous results (not shown). Six hours after infection, when L2 protein started to accumulate at ND10, colocalization of L2 with Sp100 was observed (Fig. 2). However, 12 h after infection Sp100 was no longer detectable by immunostaining, indicating that it was released from ND10. This effect was specific for L2 since neither VTF7-3 alone nor coinfection of VTF7-3 and vac33L1 encoding the HPV33 L1 capsid protein affected the association of Sp100 with ND10 (Fig. 2).

To address the question of whether Sp100 was committed to degradation or merely dispersed in the nucleoplasm, we performed Western blots using whole-cell extracts of cells infected with helper virus or helper virus and vac33L2, respectively (Fig. 3A). The extracts contained the same amount of PML, confirming that PML was unaffected by L2 expression, as previously suggested by immunostaining (Fig. 1). In contrast, the amount of Sp100 was drastically reduced in cells expressing L2 (Fig. 3A), excluding nuclear dispersal.

We next sought to analyze whether Sp100 was downregulated by L2 at the level of transcription. To address this issue, we performed Northern blots using RNA from HuTK$^-$ cells. Cells were either infected with vac33L2 and helper virus or infected with helper virus alone, and RNA was prepared 5, 10, and 24 h after infection. As shown in Fig. 3B, Sp100 mRNA was detected as a single major band of approximately 2.0 kb in all RNA preparations. At 24 h after infection a smaller RNA species was detected as well. Although vaccinia-infected cells contained somewhat less Sp100 mRNA than uninfected cells, the level of Sp100 mRNA was independent of the expression of L2. Clearly, the L2-induced reduction of the intracellular concentration of Sp100 was not caused by repression of Sp100 transcription.

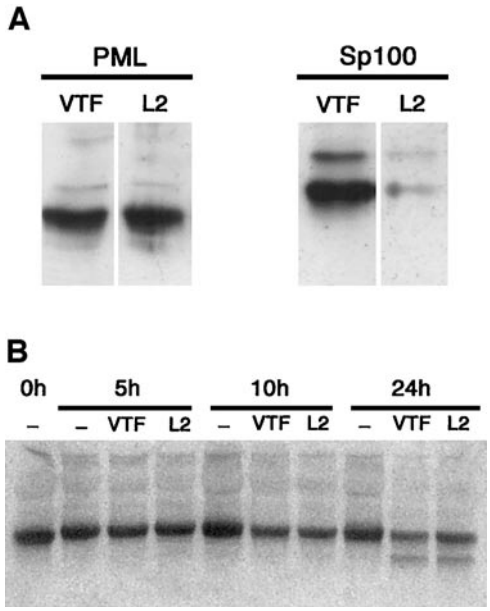

FIG. 3. Loss of Sp100 is not due to transcriptional repression. (A) HuTK$^-$ cells were infected for 24 h with VTF7-3 alone (VTF) or together with vac33L2 (L2) and subsequently processed for Western blot analysis. The blots were stained using PML-specific (PML) and Sp100-specific (Sp100) polyclonal antisera, respectively. Bound antibodies were visualized by enhanced chemiluminescence (Amersham Pharmacia). (B) Total cell RNA was prepared from uninfected HuTK$^-$ cells or from cells infected with VTF7-3 alone (VTF) or together with vac33L2 (L2) for the indicated periods of time. RNA was subsequently subjected to Northern blot analysis using a Sp100-specific probe. Radioactivity was detected with a Molecular Dynamics phosphorimager (Storm 840).

Recent studies indicate that ND10 are sites of proteolytic degradation and associate with components of the proteasome (Everett *et al.,* 1997). This suggested to us that the L2-induced diminution of Sp100 might be due to proteolysis. Addressing this question, we observed that proteasome inhibitors alone induced the dispersion of Sp100 from ND10 in the absence of L2. In addition, nuclear transport of L2 was blocked in the presence of MG132 and other proteasome inhibitors, thus preventing the use of these inhibitors to further analyze the L2-induced loss of Sp100 from ND10 (data not shown).

The transcriptional repressor Daxx is recruited into ND10 by L2

Recent studies suggest that ND10 may be involved in regulating transcriptional repression (Li *et al.,* 2000a). Since Daxx, a Fas-modulating protein that is located mainly in the nucleus, can repress transcription and bind to PML (Ishov *et al.,* 1999), we were interested to know how the accumulation of L2 affected the association of Daxx with ND10. We initially addressed this question using immunofluorescence staining of HuTK$^-$ and KH cells (Fig. 4). In the absence of L2, endogenous Daxx localized to the nucleus in diffuse and weak punctate patterns, which is in agreement with published reports and represents heterochromatin and ND10 association, respectively (Li *et al.,* 2000a). Eight hours after infection with vac33L2, Daxx was concentrating at ND10, where it colocalized with L2 (Fig. 4). Twenty hours after infection Daxx was heavily concentrated in gross L2-containing nuclear bodies (Fig. 4), suggesting L2-induced attraction of Daxx into these domains. This effect was L2-induced since neither VTF7-3 alone nor VTF7-3 and vac33L1 encoding the HPV33 L1 capsid protein affected the association of Daxx with ND10 (Fig. 4). Since the L2-mediated accumulation of Daxx in ND10 suggested that L2 can associate with Daxx, we used immunoprecipitation to verify this notion (Fig. 5). Whole-cell extracts were prepared from uninfected cells, cells infected with the VTF7-3 helper virus, and cells infected with vac33L2 and helper virus. When submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting, Daxx was detected as a 120-kDa protein in all extracts, as expected (Fig. 5). Since the amount of Daxx in the extracts was about the same, the seemingly lower level of Daxx in cells not synthesizing L2, as suggested by immunostaining (Fig. 4), was probably due to dispersal of Daxx throughout the nucleus. Immunoprecipitation of aliquots from these extracts was then carried out using an L2-specific monoclonal antibody. We used a Daxx-specific monoclonal antibody to probe for the presence of Daxx in the immunoprecipitates in Western blots. As shown in Fig. 5, Daxx was exclusively detected in the extract of vac33L2-infected cells, although it was present in all three extracts at similar concentrations. We then stripped the Daxx antibody off the membrane and reprobed the membrane with an L2-specific antibody. L2 was detected only in vac33L2-infected cells, as expected. This demonstrates that Daxx interacts with L2, either directly or via an intermediate.

Reorganization of ND10 in natural lesions

Since the nuclear architecture of terminally differentiating keratinocytes, in which papillomaviruses replicate, may not exhibit the same distinctive substructures as the cultured cells used in this work, we sought to analyze whether L2 induces the reorganization of ND10 in natural lesions. Paraffin-embedded sections of cervical intraepithelial lesions (CIN II) infected with HPV33 were stained for L2 and Sp100 (Figs. 6B and 6C). In addition, tissue classified as normal (CIN0) was stained for Sp100 (Fig. 6A). Overviews of the sections after staining with hematoxilin are also shown. Probably due to paraffin inhibition, Daxx was not detectable with the available antibody. The sections contained both enucleated keratinocytes and live cells. Sp100 was detected in nuclear dots resembling ND10 structures in normal tissue (CIN0) with the exception of stratified cells of the upper layers (Fig. 6A). L2 was visualized in the nuclei of HPV-infected cells in the upper layers but no Sp100 could be detected in these cells (Fig. 6B). Sp100 was found only in a punctate pattern in the lower layers of cells (Fig. 6B). In the intermediate layers of cells displaying weak L2-specific fluorescence, L2 colocalized with Sp100 in a punctate pattern (Fig. 6C). We assume that these cells are at the onset of L2 synthesis. This is in agreement with our observation that at the beginning of L2 expression L2 and Sp100 colocalize in ND10 of cultured cells. These results again suggest that the life cycle of this papillomavirus involves reorganization of ND10 caused by the minor capsid protein L2.

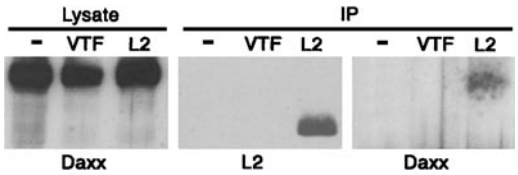

FIG. 5. L2 and Daxx coimmunoprecipitate. Nuclear extracts of uninfected HuTK$^-$ cells (−) or cells infected for 24 h with VTF7-3 alone (VTF) or together with vac33L2 (L2) were subjected to Western blot analysis using a Daxx-specific polyclonal antiserum (left). Aliquots of the same extracts were subjected to immunoprecipitation (IP) using monoclonal antibody 33L2-1 (L2, Daxx). The immunoprecipitates were analyzed by Western blot using a Daxx-specific antibody (Daxx). The antibody was subsequently stripped off the membrane, and the membrane was reprobed using 33L2-1 (L2).

DISCUSSION

We have shown in this paper that the protein composition of ND10 in human cells is profoundly altered by the minor capsid protein L2 of a papillomavirus: Sp100, a major constituent of ND10, was released and probably degraded, and Daxx, usually only partially colocalizing with ND10, was heavily concentrated at ND10, whereas PML was remarkably unaffected. We have confirmed that these structural changes were specifically induced by L2 and not caused by the vaccinia virus used as vector. Moreover, these observations were extended from cell lines to human tissue: Sp100 was absent from keratinocytes expressing L2 in HPV-infected mucosa. This is therefore the first case of a late viral protein inducing differential changes in the protein composition of ND10

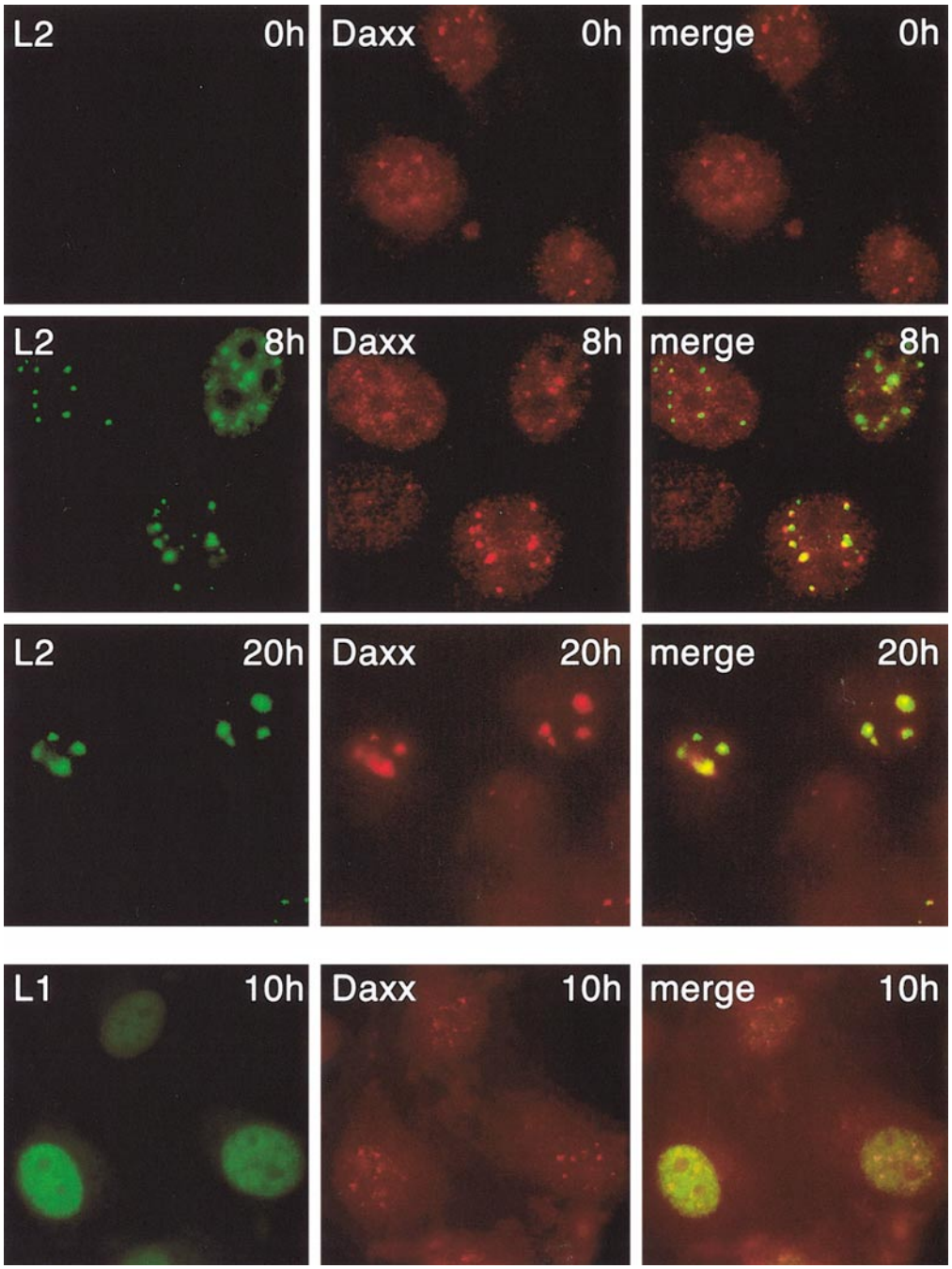

FIG. 4. L2-induced accumulation of Daxx in ND10. HuTK$^-$ cells were grown on coverslips and subsequently coinfected with VTF7-3 and vac33L2 or with VTF7-3 and vac33L1 for the indicated periods of time. Cells were stained for L2 and Daxx or for L1 and Daxx and visualized by DTAF- and Cy3-coupled secondary antibodies, respectively.

and clearly different from the observations made for large DNA viruses that completely disrupt the ND10 (Everett *et al.,* 1998; Muller and Dejean, 1999; Parkinson and Everett, 2000).

Although infections by vaccinia virus are lytic, most cells remained intact for at least 24 h. This allowed us to study the L2-induced changes of ND10 for up to 20 h. The time course of disappearance of Sp100 and acquisition of Daxx suggested that the two processes were physically uncoordinated: Daxx continued to accumulate at ND10 even after loss of Sp100. In contrast, the aquisition of Daxx was highest in cells showing the largest deposition of L2 (Fig. 5), suggesting interaction between the two proteins. This notion was confirmed by coimmunoprecipitation, suggesting association of Daxx with L2, either directly or via an intermediate. It is not clear at the

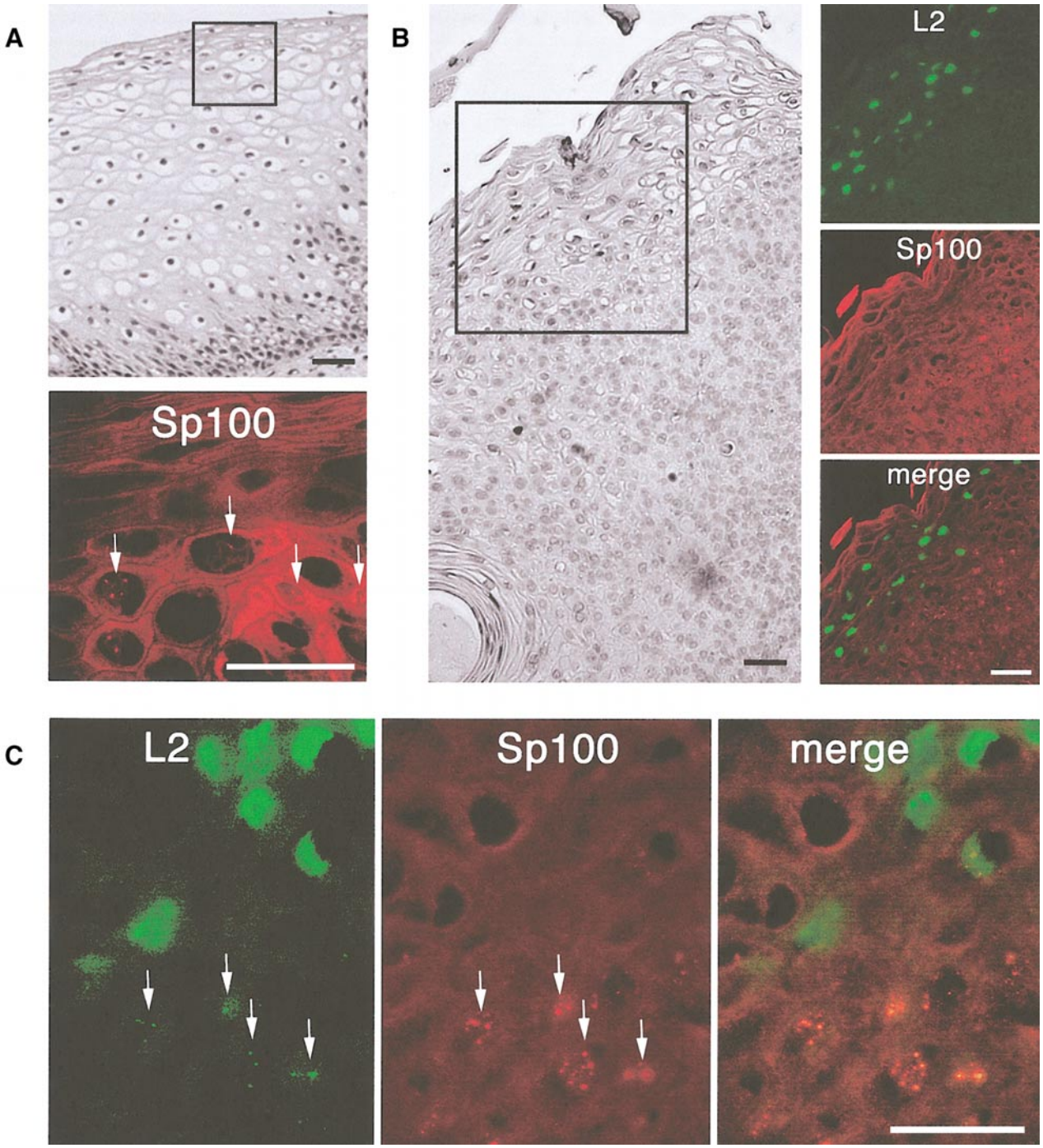

FIG. 6. Loss of Sp100 in HPV-infected lesions. Paraffin-embedded tissue sections obtained from a biopsy of normal tissue classified as CIN0 (A) and from a biopsy containing HPV-33 (B, C) were deparaffinized and were subsequently stained for Sp100 (A) or L2 and Sp100 (B, C). Overviews of the hematoxilin-stained adjacent sections are also shown. The indicated areas were enlarged for immunohistochemical analysis. (A) Sp100-positive nuclear dots in the upper layers of normal tissue are highlighted by arrows. (B) Analysis of L2 and Sp100 in the upper layers of HPV-33-infected genital mucosa. (C) Enlargement of the transition zone of B displaying the onset of L2 expression. Arrows depict regions of colocalization. Bars represent 50 μm.

moment whether accumulation of Daxx is due to an increased rate of association with ND10 or whether it is triggered by a decreased off-rate. Our observation that Sp100 was removed from cells expressing L2, whereas the steady-state level of Sp100 mRNA was unchanged, suggested proteolysis of Sp100. Specific inhibition of translation could be an alternative explanation. At the present time we cannot distinguish between these possibilities. Proteasome inhibitors like MG132 not only prevented L2 from entering into the nucleus but also caused

a redistribution of ND10-associated proteins (data not shown). Very recent data also suggest that ND10-associated proteins move to the nucleolus upon treatment with MG132 (Mattsson *et al.,* 2001).

The characterization of the virus-induced changes has implications for understanding the ND10 functions. Several viruses have been shown to disintegrate ND10 through the action of immediate-early proteins (Parkinson and Everett, 2000). Since their replication is reduced when the disintegration is inhibited, it has been postulated that ND10 store essential factors, which are released by the disintegration of the ND10 (Burkham *et al.,* 2001). However, this does not hold true for papillomavirus. The papillomavirus replication proteins E1 and E2 have previously been shown to colocalize with PML at ND10, and the synthesis of viral DNA was also localized at or near ND10 (Swindle *et al.,* 1999). Clearly no disintegration is required for replication of papillomavirus DNA. Similar results have been obtained for simian virus 40, which also requires ND10 localization for DNA synthesis (Tang *et al.,* 2000). The multiplication of papillomaviruses is coordinated with the differentiation of the keratinocytes of epithelia and mucosa. The capsid proteins L1 and L2 are synthesized only at the final stage of terminal differentiation, when the viral DNA is encapsidated as minichromosomes to form complete virions (Howley, 1996). The L2-induced structural changes of the ND10 indicate that these nuclear bodies have additional functions that need to be modified in order to allow the final assembly of papillomavirus particles.

The acquisition of Daxx to ND10 and the release of Sp100 in the final stage of papillomavirus assembly invites the following speculation. Daxx is a nuclear protein with multiple functions (Charette *et al.,* 2000; Michaelson, 2000). It inactivates transcriptional activators like Pax and ETS1 through direct interaction (Hollenbach *et al.,* 1999; Lehembre *et al.,* 2001; Li *et al.,* 2000b) and binds and relocates histone deacetylase (Li *et al.,* 2000a) causing a condensation of chromatin. Daxx may therefore play a role in the repression of viral replication and transcription. The Sp100 protein, on the other hand, has recently been shown to bind nonhistone proteins and affect chromatin dynamics (Seeler *et al.,* 1998). Since the major capsid protein, L1, is recruited to ND10 only after release of Sp100 (L.F., R.E.S., and M.S., unpublished observations) the loss of Sp100 might facilitate the packaging of the viral minichromosome.

The L2 protein may become a useful tool for the analysis of ND10 structure and function. The L2-induced reorganization of ND10 should help to elucidate its cellular function, as other viral proteins have in the past been extremely useful in unraveling the mechanisms of cell cycle control and tumorigenesis.

MATERIALS AND METHODS

Cell lines, recombinant vaccinia viruses, and antibodies

HuTK$^-$ 143B is an osteosarcoma cell line defective for thymidine kinase (Moss *et al.,* 1990). The generation of recombinant vaccinia viruses vac33L1 and vac33L2 has been described (Unckell *et al.,* 1997). VTF7-3 was kindly provided by B. Moss (Moss *et al.,* 1990). L1- and L2-specific mouse monoclonal (Sapp *et al.,* 1994; Volpers *et al.,* 1995) and rabbit polyclonal antibodies (Volpers *et al.,* 1993) have been described. The Sp100 antibody was kindly provided by H. Will. PML- and Daxx-specific antibodies were obtained from Chemicon and Santa Cruz, respectively. Proteasome inhibitors MG132 (Sigma Aldrich; 5 μM), Lactacystin (BIOMOL; 10 μM), and *N*-acetyl-leu-leu-norleucinal (ALLN) (BIOMOL; 25 μM) were diluted according to the manufacturers' suggestions.

Infection of cells

Confluent HuTK$^-$ cells were split 1:4 and grown for 24 h at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics (Life Technologies). Cells were washed once with phosphate-buffered saline (PBS) and subsequently infected with recombinant vaccinia viruses diluted in serum-free DMEM at a multiplicity of infection of 2 for each virus. After incubation for 1 h at room temperature, cells were washed once with PBS and covered with supplemented DMEM. Cells were processed for Western blotting, immunoprecipitation, and immunofluorescence after the indicated periods of time at 37°C, respectively.

Immunofluorescence

Cells grown on coverslips and infected with vaccinia viruses as described above were fixated with methanol–0.02 M EGTA (−20°C) for at least 10 min, washed twice with PBS, and blocked in 5% goat serum dissolved in PBS. Coverslips were then incubated for 1 h at 37°C with the indicated antibodies. After being washed with PBS, the coverslips were incubated for 1 h at 37°C with Cy3-conjugated Affinipure goat anti-rabbit IgG and DTAF-conjugated Affinipure goat anti-mouse IgG (Jackson ImmunoResearch Products). Coverslips were subsequently washed extensively with PBS and mounted onto slides using Vectashield mounting medium (Camon). Pictures were taken using a Leica DM RBE fluorescence microscope and a KOHU digital camera at an instrumental magnification of 630. Software for merging pictures was obtained from Applied Imaging Corp., U.S.A.

Northern blot analysis

A total of 4×10^5 HuTK$^-$ cells were seeded into six-well plates and grown overnight in supplemented

DMEM. They were subsequently infected with the indicated recombinant vaccinia viruses for 1 h at room temperature and incubated for the indicated periods of time. For each time point uninfected HuTK$^-$ cells served as control. Total RNA was isolated from the cells using the RNA extraction kit E.Z.N.A (peqlab, Erlangen, Germany). The amount of RNA was quantitated by densitometry, and similar amounts (10 μg RNA/lane) were size-fractionated in a 1.2% agarose–2.2 M formaldehyde gel and transferred to Hybond-N membrane (Amersham) using 10× SSC (modified from Fourney *et al.* (1988)). The membrane was baked for 2 h at 80°C, prehybridized for 4 h at 42°C in 6× SSC, 0.5% SDS, 5× Denhardt's solution, 50% formamide, 250 μg/ml denatured fragmented salmon sperm DNA, and hybridized with a Sp100-specific DNA. The Sp100 probe was generated by RT-PCR using the synthetic oligonucleotides 1223/1241 (5′-AGAGTGATAGGACAAGACC-3′) and 1518/1536 (5′-CGTCTGGAAACAGCAGCT-3′) according to Accession No. NM_003113 and total RNA from 293 cells. The resulting 314-bp fragment was purified from agarose gel using the JETSORB DNA extraction kit (Genomed, Bad Oeynhausen, Germany), submitted to sequence analysis, and labeled with [^{32}P]dATP (Feinberg and Vogelstein, 1983). After overnight hybridization the blot was washed three times with 2× SSC, 0.2% SDS at 42°C and three times with 0.2× SSC, 0.2% SDS at 60°C and analyzed by phosphorImaging (Molecular Dynamics, Krefeld, Germany). RNA transfer was confirmed by methylene blue staining of the membrane. The data were analyzed using Image Quant 1.2 software.

Immunoprecipitation

For immunoprecipitation, 10^6 cells, either uninfected or infected for 20 h with VTF7-3, or VTF7-3 and vac33L2, respectively, were harvested, washed once with PBS, and suspended in 1 ml of 10 mM Tris–HCl–10 mM KCl–1.5 mM $MgCl_2$–0.5% Nonidet-P40 (pH 7.5) supplemented with 10 μg/ml each of leupeptin and aprotinin. Cells were broken in a Dounce homogenizer (tight pestle), nuclei were collected, and proteins were extracted by sonication for 30 s at 40% output and 50% interval using a Branson Sonifier 250. The lysate was cleared by centrifugation and added to goat anti-mouse IgG-coated Dynabeads (Dynal) to which monoclonal antibody 33L2-1 had previously been coupled. After 2 h at 4°C at constant agitation the beads were collected and washed four times with 1 ml each of ice-cold PBS. Bound proteins were eluted with 1% SDS at 100°C and subsequently analyzed by SDS–PAGE followed by Western blot using the Daxx-specific monoclonal antibody and horseradish peroxidase-coupled secondary antibody. The signal was visualized by enhanced chemiluminescence (Amersham Pharmacia). The membrane was subsequently stripped off the antibody in 0.2 M sodium hydroxide for 10 min at room temperature, washed with H_2O and PBS–0.2% Tween for 5 min and 24 h, respectively, and reprobed using mAb 33L2-1.

Immunostaining of tissue sections

Paraffin-embedded HPV33-positive CIN II lesions, which were mounted on slides, were deparaffinized with xylol and subsequently rehydrated for 10 min each in decreasing concentrations of ethanol (90, 70, and 50%). The slides were rinsed with water, placed in a cuvette containing citric buffer, and subsequently boiled for 15 min in a microwave oven. After the slides were washed for 15 min immunostaining was performed as described above.

ACKNOWLEDGMENTS

We are grateful to H.-C. Selinka and S. Bhakdi for critically reading the manuscript and for helpful suggestions throughout the work. Sp100-specific antibody was kindly provided by H. Will. This work was supported by grants to M.S. and R.E.S. from Deutsche Forschungsgemeinschaft and Stiftung Rheinland-Pfalz für Innovation. L.F. and F.S. were supported by Graduiertenkolleg 194.

REFERENCES

Adamson, A. L., and Kenney, S. (2001). Epstein–Barr virus immediate-early protein BZLF1 is SUMO-1 modified and disrupts promyelocytic leukemia bodies. *J. Virol.* **75,** 2388–2399.

Ahn, J. H., Brignole, E. J., III, and Hayward, G. S. (1998). Disruption of PML subnuclear domains by the acidic IE1 protein of human cytomegalovirus is mediated through interaction with PML and may modulate a RING finger-dependent cryptic transactivator function of PML. *Mol. Cell. Biol.* **18,** 4899–4913.

Ascoli, C. A., and Maul, G. G. (1991). Identification of a novel nuclear domain. *J. Cell Biol.* **112,** 785–795.

Baker, T. S., Newcomb, W. W., Olson, N. H., Cowsert, L. M., Olson, C., and Brown, J. C. (1991). Structures of bovine and human papillomaviruses. Analysis by cryoelectron microscopy and three-dimensional image reconstruction. *Biophys. J.* **60,** 1445–1456.

Beaudenon, S., Kremsdorf, D., Croissant, O., Jablonska, S., Wain-Hobson, S., and Orth, G. (1986). A novel type of human papillomavirus associated with genital neoplasias. *Nature* **321,** 246–249.

Burkham, J., Coen, D. M., Hwang, C. B., and Weller, S. K. (2001). Interactions of herpes simplex virus type 1 with ND10 and recruitment of PML to replication compartments. *J. Virol.* **75,** 2353–2367.

Carvalho, T., Seeler, J. S., Ohman, K., Jordan, P., Pettersson, U., Akusjarvi, G., Carmo-Fonseca, M., and Dejean, A. (1995). Targeting of adenovirus E1A and E4-ORF3 proteins to nuclear matrix-associated PML bodies. *J. Cell Biol.* **131,** 45–56.

Charette, S. J., Lavoie, J. N., Lambert, H., and Landry, J. (2000). Inhibition of Daxx-mediated apoptosis by heat shock protein 27. *Mol. Cell. Biol.* **20,** 7602–7612.

Day, P. M., Roden, R. B., Lowy, D. R., and Schiller, J. T. (1998). The papillomavirus minor capsid protein, L2, induces localization of the major capsid protein, L1, and the viral transcription/replication protein, E2, to PML oncogenic domains. *J. Virol.* **72,** 142–150.

de The, H., Chomienne, C., Lanotte, M., Degos, L., and Dejean, A. (1990). The t(15;17) translocation of acute promyelocytic leukaemia fuses the retinoic acid receptor alpha gene to a novel transcribed locus. *Nature* **347,** 558–561.

Doucas, V. (2000). The promyelocytic (PML) nuclear compartment and transcription control. *Biochem. Pharmacol.* **60,** 1197–1201.

Dyck, J. A., Maul, G. G., Miller, W. H., Jr., Chen, J. D., Kakizuka, A., and Evans, R. M. (1994). A novel macromolecular structure is a target of the promyelocyte-retinoic acid receptor oncoprotein. *Cell* **76,** 333–343.

Everett, R. D. (2001). DNA viruses and viral proteins that interact with PML nuclear bodies. *Oncogene* **20,** 7266–7273.

Everett, R. D., Freemont, P., Saitoh, H., Dasso, M., Orr, A., Kathoria, M., and Parkinson, J. (1998). The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. *J. Virol.* **72,** 6581–6591.

Everett, R. D., and Maul, G. G. (1994). HSV-1 IE protein Vmw110 causes redistribution of PML. *EMBO J.* **13,** 5062–5069.

Everett, R. D., Meredith, M., Orr, A., Cross, A., Kathoria, M., and Parkinson, J. (1997). A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein. *EMBO J.* **16,** 1519–1530.

Feinberg, A. P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132,** 6–13.

Fourney, R. M., Miyakosh, J., Day, R. S., and Paterson, M. C. (1988). Northern blotting: Efficient RNA staining and transfer. *BRL Focus* **10,** 5–7.

Goddard, A. D., Borrow, J., Freemont, P. S., and Solomon, E. (1991). Characterization of a zinc finger gene disrupted by the t(15;17) in acute promyelocytic leukemia. *Science* **254,** 1371–1374.

Gottifredi, V., and Prives, C. (2001). P53 and PML: New partners in tumor suppression. *Trends Cell. Biol.* **11,** 184–187.

Guldner, H. H., Szostecki, C., Grotzinger, T., and Will, H. (1992). IFN enhance expression of Sp100, an autoantigen in primary biliary cirrhosis. *J. Immunol.* **149,** 4067–4073.

Hollenbach, A. D., Sublett, J. E., McPherson, C. J., and Grosveld, G. (1999). The Pax3-FKHR oncoprotein is unresponsive to the Pax3-associated repressor hDaxx. *EMBO J.* **18,** 3702–3711.

Howley, P. M. (1996). *Papillomaviridae:* The viruses and their replication. *In* "Fields Virology" (B. N. Fields, D. M. Knipe, P. M., Howley, *et al.,* Eds.), Vol. 2, pp. 2045–2076. Lippincott–Raven, Philadelphia.

Ishov, A. M., and Maul, G. G. (1996). The periphery of nuclear domain 10 (ND10) as site of DNA virus deposition. *J. Cell Biol.* **134,** 815–826.

Ishov, A. M., Sotnikov, A. G., Negorev, D., Vladimirova, O. V., Neff, N., Kamitani, T., Yeh, E. T., Strauss, J. F., III, and Maul, G. G. (1999). PML is critical for ND10 formation and recruits the PML-interacting protein daxx to this nuclear structure when modified by SUMO-1. *J. Cell Biol.* **147,** 221–234.

Kakizuka, A., Miller, W. H., Jr., Umesono, K., Warrell, R. P., Jr., Frankel, S. R., Murty, V. V., Dmitrovsky, E., and Evans, R. M. (1991). Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML. *Cell* **66,** 663–674.

Koken, M. H., Puvion-Dutilleul, F., Guillemin, M. C., Viron, A., Linares-Cruz, G., Stuurman, N., de Jong, L., Szostecki, C., Calvo, F., Chomienne, C., *et al.* (1994). The t(15;17) translocation alters a nuclear body in a retinoic acid-reversible fashion. *EMBO J.* **13,** 1073–1083.

Korioth, F., Maul, G. G., Plachter, B., Stamminger, T., and Frey, J. (1996). The nuclear domain 10 (ND10) is disrupted by the human cytomegalovirus gene product IE1. *Exp. Cell Res.* **229,** 155–158.

Lamond, A. I., and Earnshaw, W. C. (1998). Structure and function in the nucleus. *Science* **280,** 547–553.

Lehembre, F., Muller, S., Pandolfi, P. P., and Dejean, A. (2001). Regulation of Pax3 transcriptional activity by SUMO-1-modified PML. *Oncogene* **20,** 1–9.

Li, H., and Chen, J. D. (2000). PML and the oncogenic nuclear domains in regulating transcriptional repression. *Curr. Opin. Cell Biol.* **12,** 641–644.

Li, H., Leo, C., Zhu, J., Wu, X., O'Neil, J., Park, E. J., and Chen, J. D. (2000a). Sequestration and inhibition of Daxx-mediated transcriptional repression by PML. *Mol. Cell. Biol.* **20,** 1784–1796.

Li, R., Pei, H., Watson, D. K., and Papas, T. S. (2000b). EAP1/Daxx interacts with ETS1 and represses transcriptional activation of ETS1 target genes. *Oncogene* **19,** 745–753.

Mattsson, K., Pokrovskaja, K., Kiss, C., Klein, G., and Szekely, L. (2001). Proteins associated with the promyelocytic leukemia gene product (PML)-containing nuclear body move to the nucleolus upon inhibition of proteasome-dependent protein degradation. *Proc. Natl. Acad. Sci. USA* **98,** 1012–1017.

Maul, G. G., Negorev, D., Bell, P., and Ishov, A. M. (2000). Review: Properties and assembly mechanisms of ND10, PML bodies, or PODs. *J. Struct. Biol.* **129,** 278–287.

Michaelson, J. S. (2000). The Daxx enigma. *Apoptosis* **5,** 217–220.

Moss, B., Elroy-Stein, O., Mizukami, T., Alexander, W. A., and Fuerst, T. R. (1990). New mammalian expression vectors. *Nature* **348,** 91–92.

Muller, S., and Dejean, A. (1999). Viral immediate-early proteins abrogate the modification by SUMO-1 of PML and Sp100 proteins, correlating with nuclear body disruption. *J. Virol.* **73,** 5137–5143.

Negorev, D., and Maul, G. G. (2001). Cellular proteins localized at and interacting within ND10/PML nuclear bodies/PODs suggest functions of a nuclear depot. *Oncogene* **20,** 7234–7242.

Parkinson, J., and Everett, R. D. (2000). Alphaherpesvirus proteins related to herpes simplex virus type 1 ICP0 affect cellular structures and proteins. *J. Virol.* **74,** 10006–10017.

Quignon, F., De Bels, F., Koken, M., Feunteun, J., Ameisen, J. C., and de The, H. (1998). PML induces a novel caspase-independent death process. *Nat. Genet.* **20,** 259–265.

Roden, R. B., Greenstone, H. L., Kirnbauer, R., Booy, F. P., Jessie, J., Lowy, D. R., and Schiller, J. T. (1996). In vitro generation and type-specific neutralization of a human papillomavirus type 16 virion pseudotype. *J. Virol.* **70,** 5875–5883.

Sapp, M., Kraus, U., Volpers, C., Snijders, P. J., Walboomers, J. M., and Streeck, R. E. (1994). Analysis of type-restricted and cross-reactive epitopes on virus-like particles of human papillomavirus type 33 and in infected tissues using monoclonal antibodies to the major capsid protein. *J. Gen. Virol.* **75,** 3375–3383.

Seeler, J. S., and Dejean, A. (1999). The PML nuclear bodies: Actors or extras? *Curr. Opin. Genet. Dev.* **9,** 362–367.

Seeler, J. S., Marchio, A., Losson, R., Desterro, J. M., Hay, R. T., Chambon, P., and Dejean, A. (2001). Common properties of nuclear body protein Sp100 and TIF1 alpha chromatin factor: Role of SUMO modification. *Mol. Cell. Biol.* **21,** 3314–3324.

Seeler, J. S., Marchio, A., Sitterlin, D., Transy, C., and Dejean, A. (1998). Interaction of SP100 with HP1 proteins: A link between the promyelocytic leukemia-associated nuclear bodies and the chromatin compartment. *Proc. Natl. Acad. Sci. USA* **95,** 7316–7321.

Sternsdorf, T., Grotzinger, T., Jensen, K., and Will, H. (1997). Nuclear dots: Actors on many stages. *Immunobiology* **198,** 307–331.

Swindle, C. S., Zou, N., Van Tine, B. A., Shaw, G. M., Engler, J. A., and Chow, L. T. (1999). Human papillomavirus DNA replication compartments in a transient DNA replication system. *J. Virol.* **73,** 1001–1009.

Szekely, L., Pokrovskaja, K., Jiang, W. Q., de The, H., Ringertz, N., and Klein, G. (1996). The Epstein–Barr virus-encoded nuclear antigen EBNA-5 accumulates in PML-containing bodies. *J. Virol.* **70,** 2562–2568.

Szostecki, C., Guldner, H. H., Netter, H. J., and Will, H. (1990). Isolation and characterization of cDNA encoding a human nuclear antigen predominantly recognized by autoantibodies from patients with primary biliary cirrhosis. *J. Immunol.* **145,** 4338–4347.

Tang, Q., Bell, P., Tegtmeyer, P., and Maul, G. G. (2000). Replication but not transcription of simian virus 40 DNA is dependent on nuclear domain 10. *J. Virol.* **74,** 9694–9700.

Torii, S., Egan, D. A., Evans, R. A., and Reed, J. C. (1999). Human Daxx regulates Fas-induced apoptosis from nuclear PML oncogenic domains (PODs). *EMBO J.* **18,** 6037–6049.

Trus, B. L., Roden, R. B., Greenstone, H. L., Vrhel, M., Schiller, J. T., and Booy, F. P. (1997). Novel structural features of bovine papillomavirus capsid revealed by a three-dimensional reconstruction to 9 Å resolution. *Nat. Struct. Biol.* **4,** 413–420.

Unckell, F., Streeck, R. E., and Sapp, M. (1997). Generation and neutralization of pseudovirions of human papillomavirus type 33. *J. Virol.* **71,** 2934–2939.

Volpers, C., Sapp, M., Komly, C. A., Richalet-Secordel, P., and Streeck, R. E. (1993). Development of type-specific and cross-reactive serological probes for the minor capsid protein of human papillomavirus type 33. *J. Virol.* **67,** 1927–1935.

Volpers, C., Sapp, M., Snijders, P. J., Walboomers, J. M., and Streeck, R. E. (1995). Conformational and linear epitopes on virus-like particles of human papillomavirus type 33 identified by monoclonal antibodies to the minor capsid protein L2. *J. Gen. Virol.* **76,** 2661–2667.

Wang, Z. G., Ruggero, D., Ronchetti, S., Zhong, S., Gaboli, M., Rivi, R., and Pandolfi, P. P. (1998). PML is essential for multiple apoptotic pathways. *Nat. Genet.* **20,** 266–272.

Weis, K., Rambaud, S., Lavau, C., Jansen, J., Carvalho, T., Carmo-Fonseca, M., Lamond, A., and Dejean, A. (1994). Retinoic acid regulates aberrant nuclear localization of PML-RAR alpha in acute promyelocytic leukemia cells. *Cell* **76,** 345–356.

Zhong, S., Muller, S., Ronchetti, S., Freemont, P. S., Dejean, A., and Pandolfi, P. P. (2000a). Role of SUMO-1-modified PML in nuclear body formation. *Blood* **95,** 2748–2752.

Zhong, S., Salomoni, P., Ronchetti, S., Guo, A., Ruggero, D., and Pandolfi, P. P. (2000b). Promyelocytic leukemia protein (PML) and Daxx participate in a novel nuclear pathway for apoptosis. *J. Exp. Med.* **191,** 631–640.